

## Remarks

### The Amendments

#### Claims

New independent claim 13 is directed to a monitor protein for measuring protein phosphorylation. The monitor protein comprises (a) a phosphorylation region and (b) a pair of fluorescent proteins. A fluorescent protein of the pair is bound to each opposite end of the phosphorylation region. Phosphorylation of the phosphorylation region causes a change of fluorescence of the monitor protein. New claim 13 is supported by the specification which discloses “a monitor protein for measuring protein phosphorylation, the monitor protein comprising (a) a phosphorylation region comprising an amino acid residue or an amino acid sequence to be phosphorylated, and (b) a variable property region showing a property change attributed to a conformational change of a protein comprising at least the phosphorylation.” (Page 6, lines 2-6.) Claim 13 is also supported by the specification which discloses that “the variable property region is bound to each of both ends of the phosphorylation region” (page 6, lines 10-11) and the variable property region can be “proteins emitting fluorescence by interacting with each other” (page 8, lines 24-25). New claim 13 is also supported by originally filed claims 1-3.

Claim 4 has been amended to be dependent on claim 13 in place of claim 1, now canceled. Claim 4 has also been amended to recite that “the pair of fluorescent proteins is a red shifted green fluorescent protein (RSGFP) and a blue-shifted green fluorescent protein (BSGFP) of *Aequorea victoria*” in place of “the variable property comprises RSGFP and BSGFP green fluorescent proteins (GFP).” The amendment spells out the words that are abbreviated by the

well-known acronyms RSGFP and BSGFP. The amendment also clarifies claim 4 to refer to the "pair of fluorescent proteins" recited in claim 13.

Claims 5 and 9 have been amended to be dependent on claim 13 in place of canceled claims 1 and 2. Claim 9 has also been amended to recite that a change in "fluorescence" in place of a "property" is measured of the monitor protein. The amendment is supported by the specification which discloses that the variable property region or regions can be "proteins emitting fluorescence by interacting with each other." (Page 8, lines 24-25.)

#### Specification

The specification has been amended to correct grammatical and spelling errors. The specification has also been amended to spell out the words abbreviated by the well known acronyms CREB, RSGFP, (RGFP), BSGFP, and (BGFP).

None of these amendments introduces new matter.

#### The Objections to Specification

The specification has been objected to as not being in proper idiomatic English. Marked up and clean copies of a substitute specification accompany this response to correct any deficiencies. The substitute specification contains no new matter.

The specification has also been objected to because the words indicated by the acronyms CREB, RSGFP, (RGFP), BSGFP, and (BGFP) are not written out anywhere in the specification. The substitute specification also includes amendments to spell out the words indicated by each of these acronyms.

Withdrawal of these objections to the specification is respectfully requested.

#### The Objection to Claim 4

Claim 4 has been objected to for reciting "RSGFP" and "BSGFP" without spelling out the words abbreviated by the acronyms. Claim 4 has been amended to recite that RSGFP stands for "red shifted green fluorescent protein" and that BSGFP stands for "blue shifted green fluorescent protein."

Withdrawal of this objection to claim 4 is respectfully requested.

#### The Rejection of Claims 1-5 and 9 Under 35 U.S.C. § 112, Second Paragraph

Claims 1-5 and 9 have been rejected under 35 U.S.C. § 112, second paragraph as being indefinite. Claims 1-3 have been canceled. Thus the rejection of these claims has been rendered moot.

Applicants respectfully request withdrawal of the rejection as it is applied to claims 4, 5, and 9. The Office Action asserts claims 2-5 and 9 are indefinite for depending from a claim that recites "a protein comprising at least the phosphorylation region." Claims 2-5 and 9 have been amended to be dependent on claim 13 in place of claim 1. Claim 13 does not recite "a protein comprising at least the phosphorylation region."

Withdrawal of this rejection to claims 4, 5, and 9 is respectfully requested.

#### The Rejection of Claims 1-5 and 9 Under 35 U.S.C. § 112, First Paragraph

Claims 1-5 and 9 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter which was not sufficiently described in the specification. Claims 1-3 have been canceled. Thus the rejection of these claims has been rendered moot. The rejection of claims 4, 5 and 9 is

respectfully traversed.

Amended claims 4, 5 and 9 depend from new claim 13. Claim 13 is directed to a monitor protein for measuring protein phosphorylation. The monitor protein comprises (a) a phosphorylation region and (b) a pair of fluorescent proteins. A fluorescent protein of the pair is bound to each opposite end of the phosphorylation region. Phosphorylation of the phosphorylation region causes a change of fluorescence of the monitor protein. Claim 4 further recites that the pair of fluorescent proteins is a red-shifted green fluorescent protein and a blue-shifted green fluorescent protein. Claim 5 recites that the phosphorylation region comprises the amino acid sequence of SEQ ID NO:1. Claim 9 is directed to a method that employs the monitor protein.

The Office Action asserts that the claims are not adequately described because the specification fails to provide a sufficient number of identifying characteristics or representative species of the recited "variable property region" and "phosphorylation region" comprising the monitor protein. The Office Action asserts that

the term 'variable property region' describes a variable genus of chemical molecules (polypeptides, proteins and others) whose properties can be changed by phosphorylation of [the] attached phosphorylation region of the monitor protein. The only representative species of the claimed genus are two green fluorescent proteins from *Aequorea Victoria* fused to the CREB fragment of SEQ ID NO:1. Applicants failed to set forth any identifying characteristic of the other representatives of the genus.

Paper 9, page 3, line 19 to page 4, line 4. The Office Action also asserts that

it is not certain that any fusion of RSGFP and BSGFP protein from *Aequorea victoria* to any phosphorylation region will produce a monitor protein as claimed in claim 4. The claim is directed to a genus of fusion proteins, however, the only fusion protein species disclosed by Applicants consists of SEQ ID NO:1 and the RSGFP

and BSGFP. The disclosure is silent as to what is an identifying characteristic of the phosphorylation region that is critical for changes in fluorescence of the fusion protein in result of phosphorylation of its phosphorylation region.

Paper 9, page 5, lines 5-11, emphasis in original.

To satisfy the written description requirement, the specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d. 1555, 1563-1564 (Fed. Cir. 1991). The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics. *The Regents of the University of California v. Eli Lilly*, 119 F.3d 1559 (Fed. Cir. 1997); see also MPEP § 2163(II)(A)(3)(a)(ii). A specification need not disclose what is well known to those skilled in the art and preferably omits that which is well known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986).

To advance prosecution, claims 4, 5 and 9 have each been amended such that the monitor protein comprises a "pair of fluorescent proteins" in place of a "variable property region." The pair of fluorescent proteins of the monitor protein exhibits a change in fluorescence upon phosphorylation of the phosphorylation region of the monitor protein. As indicated in the Office Action, the specification discloses one species of this genus of "pair[s] of fluorescent proteins." The specification discloses that "proteins emitting fluorescence by interacting with each other

such as BSGFP and RSGFP of *Aequorea victoria*, can be preferably be used.” (Page 8, lines 24-26.) Other pairs of fluorescence proteins that can exhibit a change in fluorescence by interacting with each other were known in the art before the effective filing date of the application, September 2, 1998. See Exhibits A-B.

Tsien *et al.* (U.S. Patent 5,981,200, filed January 31, 1997; Exhibit A) teaches five pairs of these fluorescent proteins. The pairs of fluorescent proteins exhibited a change in fluorescence depending on whether a protease cleavage site engineered between the two fluorescent proteins was cleaved or not. Tsien teaches,

The emission spectrum of the intact fusion protein (FIG. 3) shows that FRET [fluorescence resonance energy transfer] is fairly efficient, because UV excitation causes substantial green emission from the acceptor S65C [a GFP containing a S65C amino acid substitution relative to wild-type GFP]. After proteolytic cleavage of the spacer, which permits the two domains to diffuse apart, the green emission almost completely disappears, whereas the blue emission from the Y66H/Y145F [a BSGFP containing amino acid substitutions Y66H and Y145F relative to wild-type GFP] is enhanced because its excited state is no longer being quenched by the acceptor. . . .

The tandem construct S65C-linker-P4 [a BSGFP having amino acid substitution Y66H relative to wild-type GFP] was exposed to enterokinase and excited at 368 nm. FRET diminished over time, demonstrating that one could detect cleavage of the linker by enterokinase. (See FIG. 5.)

The tandem construct S65T [a GFP having an S65T amino acid substitution relative to wild-type GFP]-linker-W7 [a BSGFP containing Y66W, N146L, M153T, V163A, and N212K relative to wild-type GFP] was exposed to trypsin and excited at 432 nm. Cleavage of the linker and separation of the moieties as detectable as a decrease in FRET over time. (See FIG. 6.)

The tandem construct P4-3 [a BSGFP containing amino acid substitutions Y66H and Y145F relative to wild-type GFP] - linker-W7 was exposed to trypsin and excited at 368 nm. FIG. 7. demonstrates the change in FRET resulting from cleavage.

The tandem construct W1B [a BSGFP having F64L, S65T, Y66W, N146I, M153T, V163A, and N212K amino acid substitutions relative to wild-type GFP]-linker-10c [yellow fluorescent protein having S65G, V68L, V72A, and T203Y amino acid substitutions relative to wild-type GFP] was exposed to trypsin and excited at 433 nm. FIG.8. demonstrates the change in FRET resulting from cleavage.

Column 24, lines 31-62. Thus Tsien teaches five pairs of fluorescent proteins that display a change in fluorescence depending on the ability of the fluorescent proteins to interact.

Day (*Molecular Endocrinology* (1998) 12:1410-1419; Exhibit B) fused either a BFP (having amino acid residue substitutions Y66H, Y145F relative to wild-type GFP) or a GFP (having a S65T amino acid substitution relative to wild-type GFP) to pituitary-specific transcription factor (Pit-1). (Page 1417, column 1, lines 28-30.) Formation of a Pit-1-Pit-1 dimer resulted in a change in fluorescence of the BFP/GFP pair of fluorescent proteins. Day teaches, "The results demonstrated that the acceptor signal exceeded the donor signal by approximately 2-fold, an indication of energy transfer from BFP-Pit-1 to GFP-Pit-1." (Page 1414, column 2, lines 10-13.) Thus, Day teaches a sixth pair of fluorescence proteins that can exhibit a change in fluorescence by interacting with each other.

Pairs of fluorescent proteins that exhibit a change in fluorescence depending on their interaction are disclosed in the specification and were well known in the art before the effective filing date of the application.

The "phosphorylation region" of the claimed monitor proteins is also adequately described in the specification. The specification discloses relevant identifying characteristics of the phosphorylation region, *i.e.*, its structural and chemical properties. The specification discloses that "a 'phosphorylation region' means a region comprising an amino acid residue to be

phosphorylated and capable of changing its conformation by phosphorylation.” (Page 7, lines 21-23.) Thus the specification discloses that the genus of phosphorylation regions share the structural property of changing conformation upon phosphorylation and the chemical property of being recognized and phosphorylated by a kinase. The specification also discloses a representative species of the phosphorylation region having these chemical and physical properties. The specification discloses that a “conformation[al] change was generated by phosphorylation of the CREB phosphorylation sequence, and thereby RSGFP and BSGFP at the [sic] either end could interfere [with] each other, emitting fluorescence.” (Page 18, lines 23-25.) Thus the specification discloses relevant identifying characteristics of the phosphorylation region and a representative species of a phosphorylation region. The specification therefore meets the written description requirement.

Furthermore, phosphorylation regions that (1) are recognized and phosphorylated by a kinase and (2) that induce a conformational change in a protein or a portion of the protein were known in the art before the effective filing date of the application, September 2, 1998. See Exhibits C-J below.

Chu *et al.* (*J. Biol. Chem.* (1998) 273:14649-14656; Exhibit C) teaches that phosphorylation of serine residues on the cGMP-dependent protein kinase (PKG) induces a conformational change in the protein. Chu teaches, “The data suggest that activation of the PKG by ligand binding (cGMP or cAMP) or by autophosphorylation (both Ser-63 and Ser-79) produces a similar conformational change.” (Page 14654, column 2, lines 28-31.) Chu further describes the conformational change as “similar to the elongation of PKG that is produced by cGMP binding.” (Page 14654, column 2, lines 50-51.) Thus Chu teaches a protein that



undergoes a conformational change upon phosphorylation, *i.e.*, that has the structural and chemical characteristics of the phosphorylation region described in the specification.

Barth *et al.* (*Biophysical J.* (1998) 75:538-544; Exhibit D) investigated structural changes induced in the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase using Fourier transform infrared spectroscopy following phosphorylation. Barth reported, "The spectra indicate that ATPase phosphorylation is accompanied by a conformational change involving  $\beta$ -sheet,  $\alpha$ -helical, and, likely, turn structures (see Results)." (Page 542, column 1, lines 37-39.) Barth teaches a protein that is phosphorylated and undergoes a conformational change upon phosphorylation. Thus Barth also teaches a protein that is a phosphorylation region.

Djordjevic *et al.* (*Proc. Natl. Acad. Sci. USA* (1998) 95:1381-1386; Exhibit E) performed a structural analysis of the *S. typhimurium* CheB methylesterase to determine if phosphorylation of CheB induced a conformational change. Djordjevic concluded, "Phosphorylation of the N-terminal domain, at Asp-56, distant from the methylesterase active site, leads to propagation of conformational changes to the interdomain interface. Disruption of the interface results in separation of the domains and a change in electrostatic potential in the esterase active site environment." (Page 1385, column 2, lines 17-22.) Thus Djordjevic teaches a protein that undergoes a conformational change upon phosphorylation and thus is a phosphorylation region.

Ulitzur *et al.* (*J. Biol. Chem.* (1997) 272:30577-30582; Exhibit F) used antibodies to determine whether phosphorylation induces a conformational change in mapmodulin. Ulitzur stated, "The fact that a polyclonal antibody raised against the nonphosphorylated form of the protein only poorly recognizes the phosphorylated form is consistent with significant conformational differences between the two forms." (Page 30581, column 1, lines 8-11.) Thus

Ulitzur teaches a protein that undergoes a conformational change upon phosphorylation, or has the structural and chemical characteristics of the phosphorylation region.

Zhang *et al.* (*J. Biol. Chem.* (1991) 266:2297-2302; Exhibit G) teaches that the C-terminal domain of mouse RNA polymerase II (CTD) undergoes a conformational change upon phosphorylation. Zhang found that unphosphorylated CTD (CTD<sub>a</sub>) migrated differently on a 10% SDS PAGE gel than phosphorylated CTD (CTD<sub>o</sub>). Following additional studies Zhang concluded, "The results of these hydrodynamic studies thus indicate that the electrophoretic mobility difference between CTD<sub>o</sub> and CTD<sub>a</sub> correspond to a conformational change caused by phosphorylation." (Page 2300, column 2, lines 27-30.) Thus Zhang also teaches a protein that has the structural and chemical characteristics of the phosphorylation region.

Hubbard (*EMBO J.* (1997) 16:5572-5581; Exhibit H) solved the crystal structure of phosphorylated insulin receptor tyrosine kinase (IRK) and compared it to the known crystal structure of unphosphorylated IRK. Hubbard determined that phosphorylation of IRK, at tyrosine residues, induced a conformation change in the A-loop, residues 1149-1170, of IRK. Hubbard teaches, "Upon insulin-triggered *trans*-autophosphorylation of the tyrosine residues within the A-loop, this peptide segment adopts a markedly different conformation which is stabilized by both pTyr and non-pTyr interactions." (Page 5579, column 1, lines 27-31.) Thus, Hubbard teaches that IRK and a specific peptide portion of IRK are phosphorylated. Hubbard also teaches that both undergo a conformational change upon phosphorylation.

Ma *et al.* (*J. Biol. Chem.* (1997) 272:28133-28141; Exhibit I) teaches that a polypeptide containing the R domain of cystic fibrosis transmembrane conductance regulator (CFTR) protein undergoes a conformational change following phosphorylation. Ma teaches that "when the R

domain is phosphorylated, it undergoes conformational change and interacts at a separate site in the first nucleotide binding fold to stimulate either binding or hydrolysis of ATP or transduction of the effect to open the pore.” (Page 28133, column 1, lines 25-30.) Thus Ma teaches a protein and a peptide within the protein that are phosphorylated and that undergo a conformational change upon phosphorylation. Thus, Ma teaches a phosphorylation region.

Drake *et al.* (*J. Biol. Chem.* (1993) 268:13081-13088; Exhibit J) determined that phosphorylation of CheY protein at aspartic acid residue 57 induces a conformational change. 4-fluorophenylalanine (4F-Phe) was incorporated into CheY and conformational changes were detected by measuring differences in resonance of phosphorylated and unphosphorylated CheY. Drake teaches that “phosphorylation of Asp57 triggers a long range conformational change, extending from the 4F-Phe<sup>14</sup> position to the 4F-Phe<sup>8</sup>, 4F-Phe<sup>30</sup>, 4F-Phe<sup>53</sup>, and 4F-Phe<sup>124</sup> positions on the other end of the molecule.” (Page 13087, column 2, lines 49-52.) Thus Drake teaches a protein that undergoes a conformation change upon phosphorylation, or has the structural and chemical characteristics of the phosphorylation region.

The specification discloses relevant identifying characteristics of “phosphorylation regions” and describes a species of the genus. The art, before the effective filing date of the application, taught numerous phosphorylation regions possessing the structural and chemical characteristics of a phosphorylation region.

The specification disclosure and the teachings of the art prior to the effective filing date of the application demonstrate that one of skill in the art would have recognized that applicants had possession of the claimed monitor proteins comprising a pair of fluorescent proteins and a phosphorylation region.

Applicants respectfully request withdrawal of this rejection.

The Rejection of Claims 1-5 and 9 Under 35 U.S.C. § 112, First Paragraph

Claims 1-5, and 9 are rejected under 35 U.S.C. § 112, first paragraph as not being enabled for their full scope. Claims 1-3 have been canceled. Thus the rejection of these claims has been rendered moot. The rejection of claims 4, 5, and 9 is respectfully traversed.

To satisfy the enablement requirement, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation. *In re Wright*, 999 F.2d 1557, 1561 (Fed. Cir. 1993). That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue." *In re Vaeck*, 947 F.2d 488, 495 (Fed. Cir. 1991). The test is not merely quantitative, because a considerable amount of experimentation is permissible if the experimentation is merely routine or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988).

The Office Action asserts that the claims are not enabled "because the specification, while being enabling for the variable property region consisting of two green fluorescence proteins from *Aequorea victoria* does not reasonably provide enablement for any other chemical molecule that shows a property change caused by phosphorylation of the phosphorylation region of the monitor protein." (Paper 9, page 5, lines 16-21.) The Office Action also asserts that the claims are not enabled because "the disclosure does not teach how to select [a] phosphorylation region and [a] variable property region, neither a [sic] guidance is provided as to the way both

components of the fusion protein will be fused to form the monitor protein.” (Page 7, lines 3-6.)

Enablement of a “variable property region”

As indicated above, the claims have been amended to recite that the monitor protein comprises “a pair of fluorescent proteins” in place of “a variable property region.” The pair of fluorescent proteins has the ability change fluorescence upon phosphorylation of the phosphorylation region. Claim 13, the only pending independent claim, recites “wherein phosphorylation of the phosphorylation region causes a change of fluorescence of the monitor protein.”

One of skill in the art would have been able to make and use the claimed monitor protein comprising “a pair of fluorescent proteins” without undue experimentation. The specification discloses how to make and use pairs of fluorescent proteins that undergo a change in fluorescence if their ability to interact is changed. The specification discloses that a pair of fluorescent proteins, RSGFP and BSGFP, display a change in fluorescence as a result of phosphorylation of the CREB peptide: “This indicates that [a] conformation[al] change was generated by phosphorylation of the CREB phosphorylation sequence, and thereby RSGFP and BSGFP at the either end could interfere [with] each other, emitting fluorescence.” (Page 18, lines 22-25.)

Before the effective filing date of the application, September 2, 1998, the art also taught how to make and use pairs of fluorescent proteins that undergo a change in fluorescence if the interaction of the proteins was changed.

As indicated above, Tsien (Exhibit A) and Day (Exhibit B) teach six pairs of fluorescent proteins that exhibit a change in fluorescence depending on their interaction. Tsien teaches five

pairs of these fluorescent proteins which include:

- S65C, a GFP containing a S65C amino acid substitution relative to wild-type GFP, and Y66H/Y145F, a BSGFP containing amino acid substitutions Y66H and Y145F relative to wild-type GFP;
- S65C, a GFP containing a S65C amino acid substitution relative to wild-type GFP, and P4, a BSGFP having amino acid substitution Y66H relative to wild-type GFP;
- S65T, a GFP having an S65T amino acid substitution relative to wild-type GFP, and W7, a BSGFP containing Y66W, N146L, M153T, V163A, and N212K relative to wild-type GFP;
- P4-3, a BSGFP containing amino acid substitutions Y66H and Y145F relative to wild-type GFP, and W7, a BSGFP containing Y66W, N146L, M153T, V163A, and N212K relative to wild-type GFP; and
- W1B, a BSGFP having F64L, S65T, Y66W, N146I, M153T, V163A, and N212K amino acid substitutions relative to wild-type GFP, and 10c, a yellow fluorescent protein having S65G, V68L, V72A, and T203Y amino acid substitutions relative to wild-type GFP.

Column 24, lines 31-62. Day further teaches that a BFP, containing amino acid residue substitutions Y66H, Y145F relative to wild-type GFP and a GFP, having a S65T amino acid substitution relative to wild-type GFP, exhibit a change in fluorescence depending on the ability of the pair of fluorescent proteins to interact. (Page 1414, column 2, lines 10-13.) Thus, the teachings in the specification, combined with the knowledge available in the art, demonstrate that one of skill in the art would have been able to make and use a monitor protein comprising a "pair of fluorescent proteins" capable of emitting a change in fluorescence upon phosphorylation of the monitor protein at the time the application was filed.

How to select a variable property region, a phosphorylation region and how to fuse the variable and phosphorylation regions

As discussed above, the claims have been amended to recite that the monitor protein comprises "(a) a phosphorylation region, and (b) a pair of fluorescent proteins" in place of "(a) a phosphorylation region" and "(b) a variable property region."

As discussed above, the specification and the knowledge of one of skill in the art at the time the application was filed were such that pairs of fluorescent proteins exhibiting a change in fluorescence depending on their interaction were known. One of skill in the art would also have been able to select a pair of fluorescent proteins to make the claimed monitor proteins.

The specification and the knowledge in the art at the time the application was filed were such that phosphorylation regions were also known and could have been selected by the skilled artisan. The specification discloses a CREB polypeptide that can be selected as a phosphorylation region in the monitor protein. The specification discloses that a "fusion protein derived from pETIC-ART [a plasmid encoding a RSGFP-CREB phosphorylation sequence-BSGFP protein] showed the differences in absorption wavelength." (Page 5, lines 1-2.) Thus the specification teaches one phosphorylation region that could be selected by one of skill in the art. The specification discloses another example of phosphorylation regions. The specification discloses, "As the protein to be phosphorylated, CREB transcription factor and ATF1 are exemplified." (Page 7, lines 26-29, citations omitted.)

The specification further provides guidance as to how to select a phosphorylation region that can be used in the monitor protein by disclosing examples of proteins that contain phosphorylation sites and the amino acid sequences within those proteins that are phosphorylated. The specification discloses:

Examples of kinases used for screening in the present invention (*and phosphorylation sequences thereof*) include A kinase (CREB phosphorylation sequence of SEQ ID NO:1), G kinase (RKRS\*RAE; histone), AMP activation protein kinase (HMRSAMS\*GLHLVKRR; acetyl CoA carboxylase), calmodulin-dependent protein kinase II (PLRRTLS\*VAA; glycogen synthase) smooth muscle myosin light chain kinase (KKRAARATS\*NVFA; myosin light chain), phosphorylase

kinase (KRKQIS\*VRGSL; phosphorylase), C kinase (VRKRT\*LRRL; EGF receptor), v-Abl (RRLIEDAEY\*AARG; RR60<sup>SRC</sup>), EGF receptor protein kinase (RREELQDDY\*EDD; erythrocyte band III), but are not limited thereto.

Page 11, lines 5-14, emphasis added. The specification discloses numerous examples of proteins and amino acid sequences that are phosphorylated and can comprise a phosphorylation region in the claimed monitor protein.

It would not require undue experimentation to test if any of these sequences can be used in a monitor protein. The specification teaches how to test if an amino acid sequence can be used as a phosphorylation region, and this test would be routine for one of skill in the art to perform. The specification teaches that this test is performed "by inserting [the] phosphorylation sequence between RSGFP and BSGFP and performing both phosphorylation measurements using [ $\gamma$ -<sup>32</sup>P]ATP and those based on fluorescence change." (Page 19, lines 10-13.) If the inserted phosphorylation sequence can be phosphorylated in the  $\gamma$ -<sup>32</sup>P assay but no fluorescence change is observed in the fluorescence assay, it means that the phosphorylation sequence is phosphorylated but does not induce a fluorescence change and thus cannot be used as a phosphorylation region in a monitor protein. The specification provides detailed instructions for performing the [ $\gamma$ -<sup>32</sup>P]ATP assay in Example 4: "Measurement of phosphorylation using  $\gamma$ -<sup>32</sup>P." (See page 17, lines 7-33.) The specification also provides detailed instruction for performing fluorescence measurements in Example 5: "Measurement of phosphorylation by fluorescence change." (See page 17, line 35 to page 19, line 13.) Using these specification teachings, one of skill in the art could readily test if a phosphorylation sequence can be used as a phosphorylation region.

Furthermore, before the effective filing date of the application, the art taught proteins and



polypeptides that can be used as phosphorylation regions in the claimed monitor protein.

(Exhibits C-J.) Each of these proteins or polypeptides contains at least one amino acid residue that is phosphorylated and each of these proteins or polypeptides undergoes a conformational change upon phosphorylation of the amino acid residue(s):

- Chu teaches that phosphorylation of serine residues on the cGMP-dependent protein kinase (PKG) induces a conformational change in the protein. (Exhibit C.)
- Barth teaches that a structural change is induced in the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase following phosphorylation. (Exhibit D.)
- Djordjevic teaches that phosphorylation of *S. typhimurium* CheB methylesterase induces a conformational change in the protein. (Exhibit E.)
- Ulitzur teaches that phosphorylation induces a conformation change in mapmodulin. (Exhibit F)
- Zhang teaches that the C-terminal domain of mouse RNA polymerase II (CTD) undergoes a conformational change upon phosphorylation. (Exhibit G.)
- Hubbard teaches that IRK and a specific peptide portion of IRK are phosphorylated. Hubbard teaches that phosphorylation of IRK or the peptide portion of IRK induces a conformational change. (Exhibit H.)
- Ma teaches that a polypeptide containing the R domain of cystic fibrosis transmembrane conductance regulator (CFTR) protein undergoes a conformational change following phosphorylation. (Exhibit I.)
- Drake teaches that phosphorylation of CheY protein at aspartic acid residue 57 induces a conformational change. (Exhibit J.)

Thus the art, before the effective filing date of the application, taught proteins and polypeptide sequences that could be used as phosphorylation regions, *i.e.*, that undergo a conformational change upon phosphorylation.

The specification discloses preferred phosphorylation regions that can be used to make the monitor protein. The specification also discloses proteins and polypeptide sequences that can be phosphorylated and methods of testing whether these sequences can be used in a monitor protein. These methods would have been routine for one of skill in the art to perform. The art, before the effective filing date of the application, taught protein and peptide sequences that when

phosphorylated induce a conformational change in the protein or peptide. Thus, provided with the specification disclosure and the teachings of the art at the time the application was filed, one of skill in the art could have selected a phosphorylation region for use in the claimed monitor protein.

The specification and the knowledge in the art at the time the application was filed were also such that the skilled artisan could have fused the phosphorylation region to the pair of fluorescent proteins without undue experimentation. The specification discloses that monitor protein is a fusion protein of the phosphorylation region and the pair of fluorescent proteins: “‘Property variable regions’ (*e.g.*, a pair of fluorescent proteins) can separately [be] present [in the monitor protein] as long as [they] form a fusion protein with a phosphorylation region.” (Page 8, lines 12-13.) Methods of producing fusion proteins were well known and routinely practiced in the art at the time the application was filed. The Office Action acknowledges that “synthesis of hybrid molecules [was] well known in the art.” (Paper 9, page 6, lines 17-18.) One of skill in the art would thus have been able to fuse the phosphorylation region and the pair of fluorescent proteins that comprise the monitor protein without having to resort to undue experimentation.

Applicants respectfully request withdrawal of this rejection.

#### The Rejection of Claims 1, 2, and 9 Under 35 U.S.C. § 102(b)

Claims 1, 2, and 9 are rejected under 35 U.S.C. § 102(b) as being anticipated by Tsien *et al.* (U.S. Patent 5,925,558). Claims 1 and 2 have been canceled. Thus the rejection of these claims has been rendered moot. Applicants respectfully traverse the rejection of claim 9.

To reject claims as anticipated, each and every element as set forth in the claim must be found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Tsien does not teach each and every element recited in amended claim 9.

Amended claim 9 is directed to a method for measuring phosphorylation ability of a test protein. The method comprises a step of reacting the test protein with a monitor protein and a step of measuring fluorescence of the monitor protein. The monitor protein comprises (a) a phosphorylation region and (b) a pair of fluorescent proteins. A fluorescent protein of the pair is bound to each opposite end of the phosphorylation region. Phosphorylation of the phosphorylation region causes a change of fluorescence of the monitor protein.

Tsien is cited as teaching a modified fluorescent protein that contains a phosphorylation site for a protein kinase and that exhibits different fluorescent characteristics depending on whether it is phosphorylated. (Paper 9, page 8, lines 13-17.) Tsien is also cited as teaching a method for determining whether a sample has phosphorylation ability. (Paper 9, page 8, lines 18-19.)

Tsien does not explicitly or inherently teach the monitor protein of claim 9. The monitor protein of claim 9 comprises "(a) a phosphorylation region and (b) a pair of fluorescent proteins." The protein taught by Tsien contains only a single fluorescent protein moiety and a phosphorylation site for a kinase: "Fluorescent protein substrates for a protein kinase comprise a fluorescent protein moiety and a phosphorylation site for a protein kinase." (Column 4, lines 25-27.) Thus, Tsien teaches a fluorescent protein substrate that contains one fluorescent protein moiety. Tsien does not expressly or inherently teach a protein comprises "a pair of fluorescent

proteins.” Thus, Tsien does not expressly or inherently teach each and every element recited in claim 9 and does not anticipate claim 9.

Applicants respectfully request withdrawal of this rejection to claim 9.

#### The Rejection of Claim 5 Under 35 U.S.C. § 103(a)

Claim 5 has been rejected under 35 U.S.C. § 103(a) as unpatentable over Tsien *et al.* (U.S. Patent 5,925,558) in view of Hagiwara *et al.* (*Mol. Cell. Biol.* (1993) 13:4852-4859).

Applicants respectfully traverse.

The Patent Office has the burden of establishing a *prima facie* case of obviousness. (MPEP § 2142.) To reject a claim as *prima facie* obvious three criteria must be met:

First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

MPEP § 1243. The rejection fails to meet the third criterion, *i.e.*, the combination of references fails to teach or suggest all the claim limitations. Thus the *prima facie* case of obviousness must fail.

Amended claim 5 depends from claim 13. Claim 13 is directed to a monitor protein for measuring protein phosphorylation. The monitor protein comprises (a) a phosphorylation region and (b) a pair of fluorescent proteins. A fluorescent protein of the pair is bound to each opposite end of the phosphorylation region. Claim 5 further recites that the phosphorylation region comprises the amino acid sequence of SEQ ID NO:1.

Tsien is cited as teaching proteins containing a phosphorylation region recognized by protein kinase A and a fluorescent protein from *Aequorea*. (Paper 9, page 10, lines 6-8.)

Hagiwara is cited as teaching that protein kinase A phosphorylates CREB at amino acid residue 133, a serine residue, which is contained in the amino acid sequence of SEQ ID NO:1. (Paper 9, page 10, lines 11-12.) The Office Action asserts that it would have been obvious for one of ordinary skill in the art to modify Tsien's modified fluorescent protein to contain a fragment of transcription factor CREB comprising serine residue 133, *e.g.*, SEQ ID NO:1, as taught by Hagiwara, because the CREB peptide comprising serine residue 133 is a substrate of protein kinase A. (Paper 9, page 10, lines 16-18.)

The combination of Tsien and Hagiwara fails to render claim 5 obvious because it fails to teach or suggest all the elements recited in independent claim 13, from which claim 5 depends. The combination of Tsien and Hagiwara does not teach or suggest a monitor protein that comprises "a pair of fluorescent proteins" as recited in claim 13. As indicated above, Tsien teaches a fluorescent protein that contains phosphorylation sequences for protein kinases. Tsien teaches, "Fluorescent protein substrates can be made by modifying the amino acid sequence of an existing fluorescent protein to include a phosphorylation site for a protein kinase." (Column 12, lines 25-28.) Tsien does not teach a protein that contains a phosphorylation site for a kinase and "a pair of fluorescent proteins." Tsien also does not suggest a protein that comprises "a pair of fluorescent proteins."

Hagiwara teaches a fragment of CREB containing amino acid residues 128 to 141. The fragment was used to detect protein kinase A phosphorylation activity in cell extracts. Hagiwara teaches that "we measured PKA [protein kinase A] activity in PC12 cell extracts, using a CREB

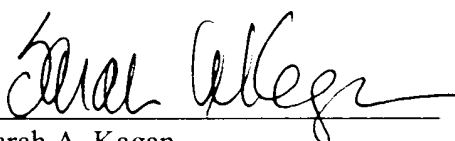
peptide (amino acids [aa] 128 to 141) as a test substrate (not shown), and calculated the level of cAMP-dependent PKA activity of PC12 cells to be 1.2  $\mu$ M, . . ." (Page 4855, column 1, lines 1-5.) Hagiwara does not teach or suggest fusion of any protein, including a fluorescent protein, to the CREB peptide. Thus Hagiwara does not teach or suggest a protein comprising a CREB peptide and "pair of fluorescent proteins" as recited in claim 13.

The combination of Tsien and Hagiwara does not teach or suggest a protein that comprises "a pair of fluorescent proteins" as recited in independent claim 13. Thus, the combination of Tsien and Hagiwara fails to teach or suggest all the limitations recited in claim 13 and cannot be used to render claim 13 obvious. If an independent claim is nonobvious under 35 U.S.C. 103, then any claim depending therefrom is nonobvious. *In re Fine*, 837 F.2d 1071 (Fed. Cir. 1988). Claim 5 depends from claim 13. Thus, claim 5 is also allowable over the combination of Tsien and Hagiwara.

Applicants respectfully request withdrawal of this rejection to claim 5.

Respectfully submitted,

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## DESCRIPTION

### MONITOR PROTEIN FOR MEASURING PROTEIN PHOSPHORYLATION

#### 5    Technical Field

The present invention relates to a protein for measuring ~~an activity of phosphorylating a protein~~ phosphorylation and a nucleic acid encoding the protein.

#### 10    Background Art

Ingenious switch mechanisms are present in ~~a living body of an organism~~ organisms, and these switch mechanisms control, for example, differentiation, development, protective ~~response~~ responses, metabolic activity, etc. ~~As one of these A~~ known switch ~~mechanisms, a mechanism is the phenomenon called of~~ protein  
15    phosphorylation ~~is known~~ (Hunter T., 1991, Methods Enzymol. 200: 3-37). Phosphorylation is mediated by an enzyme. ~~Specifically, this~~ The enzyme introduces a phosphate group to a substrate protein ~~to activate this~~ and activates the substrate protein. ~~This~~ The activated substrate protein induces diverse reactions, for instance, regulation of gene expression and cellular proliferation.

20        Recently, a cascade mechanism, called cellular signal transduction, has been uncovered. ~~which is a base~~ Cellular signal transduction is the basis for diverse reactions in a living body, including cellular development and differentiation, ~~have been uncovered. In this cellular signal transduction pathway, the above~~ The phosphorylation reaction has been shown to play an important role as one of the  
25    switch mechanisms which determine the on-state and off-state of the pathway cellular signal transduction pathway (Hunter T., 1995, Cell 80: 225-236).

So far, ~~it has been revealed that multiple pathways are present in~~ have been identified as signal transduction pathways. For instance, a relatively simple pathway

through that is a signal transduction pathway is one in which an extracellular information, such as a hormone, directly passes through a cell membrane to be and is transmitted into the inside of a cell, ~~such as a hormone, is one of signal transduction pathways, while~~ More recently, complex pathways through which a given information is transmitted into a cell by communication ~~among~~ along multiple molecules have been discovered.

One ~~An~~ example ~~is a~~ signal transduction pathway is mediated by a G protein (Gilman, A. G. , 1987, Annu. Rev. Biochem. 56: 615-649). ~~In this pathway, A signal for initiating the transduction pathway is~~ binding of a specific substance to a receptor formed on a cell membrane, ~~is a signal for initiating the transduction pathway. On the other hand,~~ G protein conjugated ~~with~~ to the receptor in a cell then elevates the intracellular concentration of cyclic-adenosine mono phosphate (cAMP), ~~which is a~~ second messenger. A specific kinase protein phosphorylates A kinase ~~by~~ using the increased concentration of cAMP. It is proposed that phosphorylation of A kinase generated in this manner, namely ~~an~~ by activation, is transmitted to, for example, the nucleus, as a signal, inducing ~~the~~ expression, such as for a protein necessary for an *in vivo* response at the final stage of the cascade ~~response~~. Other ~~than this~~ signal transduction ~~pathway mediated by G protein~~ pathways are known and include, for instance, a transduction pathway based on a cell surface receptor and MAP kinase (Madhani, H. D. et al., 1998, Trends Genet. 14: 151-155) and ~~a transduction pathway based on~~ a cell membrane phospholipid and C kinase (Weinstein, I. B. et al., 1997, Adv. Exp. Med. Biol. 400A: 313-321) ~~are known,~~ indicating that protein phosphorylation ~~plays an~~ is important ~~role as a switch for activating that activates a~~ pathway in all cases. Many studies, have suggested, however, that the presently



known signal transduction pathways ~~known at present~~ are only a part of the diverse transduction pathways expected to be present in a cell.

Thus, uncovering signal transduction pathways is important ~~in revealing~~ to reveal the various mechanisms in life of activities in life. Substances involved in  
5 novel ~~Novel~~ transduction pathways, and ~~substances involved in~~ known transduction pathways have been searched for and the functions of these substances have been analyzed. ~~Among these,~~ Studies of signal transduction pathways have included analyses on of the phosphorylation reaction as playing an important role as a switch mechanism in signal transduction, search searches for enzymes carrying on that  
10 participate in this reaction, and such, have been widely studied.

Enzymes ~~carrying on such that perform~~ protein phosphorylation ~~reaction~~ reactions have mainly been searched ~~mainly~~ by comparing homology ~~with genes encoding of known protein kinases with other genes~~. ~~In a kinase family which phosphorylates a tyrosine residue, a~~ A consensus sequence in this family is known has  
15 been identified in the family of protein kinases that phosphorylates a tyrosine residue (Hunter T. et al., 1984, Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17: 443-455), ~~and thus~~ Thus, for example, screening libraries can be screened by using a probe designed based on this consensus sequence enables searching a novel enzyme. ~~Whether an~~ An enzyme newly discovered in this manner ~~has an activity to~~  
20 phosphorylate a protein is mainly analyzed for protein phosphorylation activity mainly by in vitro analysis system using <sup>32</sup>P isotope (D. Grahame Hardie, Eds. "Protein Phosphorylation: A Practical Approach," 1993, Oxford University Press). Specifically, a searched enzyme, a substrate protein (or a known peptide) comprising phosphorylation region, and a <sup>32</sup>P phosphate are mixed to determine whether the <sup>32</sup>P is

added to the substrate protein (or the peptide), by monitoring radioactivity of a reaction product.

Conventional methods for analyzing protein phosphorylation, ~~however,~~ require the use of radioactive isotopes, such as  $^{32}\text{P}$ , limiting experimental facilities and  
5 such, requiring a careful experimental manipulation, and thus making the manipulation cumbersome. In addition, these kinds of experiments ~~accompany~~ generate radioactive waste of radioactive isotopes, and therefore, are not preferable for the natural environment. Moreover, the ~~above~~ above-described analytical method does not ~~enable~~ directly ~~measuring the~~ measure intracellular phosphorylation activity,  
10 and thus, ~~has a problem of giving~~ is problematic because it only provides results measured ~~under the~~ in an artificial environment.

To solve these problems ~~in in vitro analysis system,~~ an in vivo measurement system ~~has been~~ was developed. The method ~~does~~ did not directly measure phosphorylation, ~~but measures~~ The method measured protein phosphorylation  
15 ~~reaction by~~ indirectly using cellular proliferation induced by phosphorylation as an indicator. More specifically, in this analysis system, ~~a cellular proliferation rate is measured by uptake of  $^3\text{H}$  thymidine uptake into a cell measured, and, based on the cellular proliferation rate,~~ Based on the cellular proliferation rate, phosphorylation ~~reaction at the~~ an early stage in the signal transduction pathway ~~is~~ was measured. In  
20 this method, ~~however,~~ it is difficult to rapidly measure ~~rapid measurement of~~ phosphorylation ~~reaction is difficult~~ because ~~the~~ an activity at the end of transduction pathways, ~~namely,~~ cellular proliferation, is measured as an indicator. Moreover, the method is not suitable for measuring the timing of ~~occurrence of~~ the phosphorylation reaction and such.

~~On the other hand, an~~ An analysis system ~~by measuring that measures~~ calcium concentration *in vivo* has ~~been~~ recently been reported (Miyawaki, A. et al., 1997, Nature 388: 882-887). This system, ~~in which~~ uses a fusion protein. The fusion protein contains ~~in which~~ green fluorescent proteins (GFP) of luminous jellyfish, *Aequorea victoria*, ~~are~~ fused to both ends of a calmodulin protein. ~~is used for measurement, utilizes the characteristic that fluorescence~~ Fluorescence is emitted when the GFP proteins at the both ends approach and interact with each other. ~~More specifically, binding of~~ Thus, when a certain amount of calcium is bound to the central calmodulin protein, the ~~alters~~ conformation of the calmodulin protein is altered, and the GFP proteins at the both ends interact with each other to emit fluorescence. *In vivo* calcium concentration ~~can be~~ is determined by measuring the emitted light. ~~In the~~ Thus, a superior method that measurement of measures calcium concentration, ~~such a superior method in which does not use radioactive isotopes are not used and that is applicable to the in vivo measurement, has been developed. However, in the above~~ above-described protein phosphorylation reaction, ~~development of such a system is behind~~ has not been developed.

#### Disclosure of the Invention

20       An objective of the present invention is to establish ~~an analysis~~ a system for to analyze protein phosphorylation, ~~the analysis system~~ in which radioactive isotopes are not used and that is applicable to ~~the~~ *in vivo* measurement.

      The present inventors first obtained genes encoding the cAMP response element binding protein (CREB) ~~CREB~~ phosphorylation sequence (Gonzalez, G. A. et al., 1989, Cell 59: 675-680) and the ~~kemptide~~ phosphorylation sequence (Kemp B. E. et al., 1977, J. Biol. Chem. 252: 4888-4894), which are ~~as~~ representative examples of known amino acid sequences that are ~~known to be~~ phosphorylated, ~~and prepared~~

plasmid "~~pETIC-ART~~" and "~~pETIC-Kempart~~", respectively, by inserting each gene  
The genes were inserted between a red-shifted green fluorescent protein (RSGFP)  
RSGFP site (also called RGFP) and a blue-shifted green fluorescent protein (BSFGP)  
BSGFP site (also called BGFP) of a plasmid pETIC (Romoser V. A. et al., 1997, J.  
5 Biol. Chem. 272: 13270-13274) to prepare plasmids "~~pETIC-ART~~" and "~~pETIC-  
Kempart~~," respectively. Both plasmids can express a fusion protein comprising a  
structure of "RSGFP- a protein phosphorylation sequence- BSGFP" and are different  
only in the protein phosphorylation sequences. As a negative control in the  
phosphorylation reaction experiment, pETIC-1 was used. This plasmid can express a  
10 fusion protein comprising the structure composed of "RSGFP- a calmodulin binding  
site - BSGFP" (Romoser V. A. et al., J. Biol.Chem., 1997, 272: 13270-13274).

*E. coli* BL-21 (DE3) was transformed with pETIC-ART, pETIC-Kempart, or  
pETIC-1, and the above fusion proteins were extracted from the microbial cells after  
incubation.

15 Analysis by using an *in vitro* analysis system with  $^{32}\text{P}$  isotope found that the  
fusion proteins derived from pETIC-ART and pETIC-Kempart were phosphorylated,  
but the fusion protein derived from pETIC-1 was not.

~~Whether A change in fluorescence changed or not was examined in the case~~  
~~that the reaction was carried out~~ was determined using a non-radioactive phosphate as  
20 a substrate, ~~without  $^{32}\text{P}$  isotope. As a result, depending on the presence or absence of~~  
~~phosphorylation reaction, the~~ The fusion protein derived from, pETIC-ART showed  
the differences in absorption wavelength depending on whether phosphorylation  
occurred with the nonradioactive phosphate. , ~~however, the presence or absence of~~  
~~phosphorylation in the~~ The fusion proteins derived from pETIC-Kempart or pETIC-1

did not ~~lead to~~ show differences in absorption wavelength depending on whether phosphorylation occurred. These results uncovered the following two points.

1. Phosphorylation ~~in~~ of the fusion protein derived from "pETIC-ART" generates protein conformational change necessary for generating changes in GFP  
5 fluorescence.

2. The fusion protein derived from "pETIC-Kempart" is phosphorylated, however, this phosphorylation does not generate protein conformational change necessary for generating changes in GFP fluorescence.

Specifically, the present inventors have shown that, in some cases, protein  
10 phosphorylation generates a conformational change in a protein. ~~The~~ Using the combination of two kinds of GFP proteins, as described above, is provides an example for detecting conformational change as a property change, ~~and Thus,~~ it is obviously possible that the same system can be constructed for proteins having other properties, etc.

15 ~~Then Thus,~~ fusion of a protein in which containing a region for monitoring a property change, ~~namely~~ a variable property region, and a region to be phosphorylated, ~~namely~~ a phosphorylation region, ~~are fused~~ can be used as a protein for monitoring protein phosphorylation (a monitor protein). ~~This case~~ The use of a monitor protein does not require the use of radioactive isotopes and is applicable to *in*  
20 *vivo* measurement. In fact, the present inventors have successfully monitored phosphorylation in real time in living cells into which a vector expressing a monitor protein has been introduced.

Such a monitor protein can easily monitor protein phosphorylation, ~~thereby~~ and therefore can be used ~~for~~ in a method for screening for a novel kinase and such.

Moreover, using this system, a compound which stimulates or inhibits phosphorylation can be screened.

The finding that some protein kinases generate a conformational change in a substrate, and others do not ~~enable determining~~ indicates that monitor proteins can  
5 also be used to determine whether protein phosphorylation generates a conformational change, or not by using The methods would use both an existing *in vitro* analysis system with <sup>32</sup>P isotope, and a method using the property change of a monitor protein. More specifically, the present invention relates to:

- (1) a monitor protein for measuring protein phosphorylation, the monitor  
10 protein comprising (a) a phosphorylation region comprising an amino acid residue or an amino acid sequence to be phosphorylated, and (b) a variable property region showing a property change attributed to a conformational change of a protein comprising at least the phosphorylation region, which conformational change is caused by phosphorylation of the amino acid residue or the amino acid sequence;
- 15 (2) the monitor protein of (1) , wherein the variable property region is a protein that emits fluorescence;
- (3) the monitor protein of (1) or (2), wherein the variable property region is bound to each of both ends of the phosphorylation region;
- (4) the monitor protein of (3), wherein the variable property region  
20 comprises RSGFP and BSGFP which are comprised in green fluorescent protein (GFP) of *Aequorea victoria*;
- (5) the monitor protein of any one of (1) to (4), wherein the phosphorylation region comprises the amino acid sequence of SEQ ID NO: 1
- (6) a nucleic acid encoding the monitor protein of any one of (1) to (5);
- 25 (7) an expression vector carrying the nucleic acid of (6);

(8) a method for measuring phosphorylation ability in a cell by introducing the monitor protein of any one of (1) to (5), the nucleic acid of (6), or the expression vector of (7) into the cell;

(9) a method for measuring phosphorylation ability of a test protein, the  
5 method comprising reacting the test protein with the monitor protein of any one of (1) to (5), and measuring a property change of the monitor protein;

(10) a method for screening a kinase, the method comprising:

- (a) reacting a test protein with the monitor protein of any one of (1) to (5),
- 10 (b) measuring the property change of the monitor protein, and
- (c) selecting the test protein which alters the property of the monitor protein;

(11) a method for screening a compound which stimulates or inhibits phosphorylation, the method comprising:

15 (a) contacting, in the presence of a test sample, a kinase with the monitor protein of any one of (1) to (5), the monitor protein comprising a phosphorylation region to be phosphorylated by the kinase,

(b) measuring the property a change of the monitor protein, and

(c) selecting a compound which stimulates or inhibits the property  
20 change in comparison with the property change in the absence of the test sample; and

(12) a method for screening a compound which stimulates or inhibits phosphorylation, the method comprising:

(a) preparing a cell into which the expression vector of (7) is introduced,

(b) measuring, in the presence of a test sample, the property change of a monitor protein expressed in the cell, and

(c) selecting a compound which stimulates or inhibits the property change in comparison with the property change in the absence of the test sample.

5

The present invention is illustrated in detail below.

1. A monitor protein for measuring protein phosphorylation

A monitor protein for measuring protein phosphorylation (a monitor protein,  
10 hereafter) is a protein comprising a "phosphorylation region" and one or more "variable property regions."

Herein, a "phosphorylation region" means a region comprising an amino acid residue to be phosphorylated and capable of changing its conformation by phosphorylation of the amino acid residue. Among the ~~following~~ proteins known to  
15 be phosphorylated, those capable of showing conformational change by phosphorylation can be used as a phosphorylation region. As the protein to be phosphorylated, exemplary examples are CREB transcription factor (Hagiwara M. et al., 1993, Mol. Cell. Biol. 13: 4852-4859) and ATF1 (Shimomura, A. et al., 1996, J. Biol. Chem. 271: 17957-17960) ~~are exemplified~~.

20 ~~For example, a~~ A conformational change can even be induced ~~even~~ in a protein, for example, which does not usually undergo any conformational change by a phosphate group-specific antibody. Therefore, a phosphate group-specific antibody can be combined with another property variable region.

~~A~~ The phosphorylation region is preferably a partial sequence of a protein  
25 comprising an amino acid residue to be phosphorylated ~~can be preferably used as well~~



as a but can also be the full length of the protein comprising the phosphorylation region. For instance, in the case of CREB transcription factor, it is known that the serine at 133rd residue at amino acid 133 is phosphorylated by protein kinase A. Therefore, any partial sequence of CREB transcription factor can be used as long as it  
5 contains the ~~133rd~~ serine residue at amino acid 133 and is capable of being phosphorylated. For example, as the partial sequence, an amino acid sequence of SEQ ID NO: 1 can be selected. As an "amino acid residue to be phosphorylated," for example, tyrosine, threonine, and so on can be used other than serine.

Any amino acid sequence can be used as a "variable property region" as long  
10 as its phosphorylation can be easily determined. An example is GFP.

"Property variable regions" can be separately present as long as ~~forming~~ they form a fusion protein with a phosphorylation region. A monitor protein can ~~be~~ preferably be constructed as a "measurement protein pair" provided at both ends of a phosphorylation region. More specifically, an amino acid residue in a  
15 phosphorylation region is phosphorylated to alter the conformation of the phosphorylation region, and a measurement protein pair prepared at both the ends of the phosphorylation region interacts to show a measurable property. For example, each component can be designed as follows.

Any "measurement protein pair" provided at both ends of the above  
20 phosphorylation region can be used as long as ~~showing the pair displays~~ a measurable property ~~by interacting with each other after interaction~~ due to conformational change of the ~~above~~ phosphorylation region. For example, proteins emitting fluorescence by interacting with each other, such as BSGFP and RSGFP of *Aequorea victoria*, can ~~be~~ preferably be used.

Any desirable functional regions can be added to a monitor protein other than the above "phosphorylation region" and "variable property region." Examples include a nuclear localization signal to transport a monitor protein to the nucleus ~~for measuring and measure~~ phosphorylation in the nucleus (Goldfarb, D. S. et al., 1986, Nature 322: 641-644) and a marker ~~which is as~~ an indicator ~~for the introduction of~~ that a monitor protein is introduced into a cell (Heim, R. et al., 1995, Nature 373: 663-664).

## 2. Production of a monitor protein

Any methods for producing the above monitor protein can be used. For example, a monitor protein can be produced by expressing a nucleic acid encoding the monitor protein in any host cell, such as *E. coli*. More specifically, this "nucleic acid encoding a monitor protein" is constructed so that a measurement protein pair and a phosphorylation region can be expressed as a fusion protein. The nucleic acid is produced by ligating a nucleotide sequence encoding the above variable property region and a nucleotide sequence encoding the phosphorylation region in the same reading frame. A monitor protein can be amplified and produced by ligating the nucleic acid encoding the monitor protein constructed above to any vector, introducing the vector into an appropriate host cell, and expressing the ~~nuclei~~ nucleic acid. A monitor protein produced in this manner can be used for measuring *in vitro* or *in vivo* phosphorylation directly, or preferably be used after isolation and purification.

Any combination of a vector and a host cell can be used for producing a monitor protein, and the nucleic acid encoding a monitor protein can be ligated downstream of a promoter with high expression activity for improving monitor

protein expression. Such a promoter and the like can be arbitrary selected from known promoters and used.

3. A nucleic acid encoding a monitor protein

The above "nucleic acid encoding a monitor protein" can include ~~those contain~~  
5 ~~another sequence~~ other sequences as long as the nucleic acid encoding encodes the  
monitor protein. ~~Another sequence includes~~ These other sequences include a sequence  
~~effectively expressing that causes effective expression of~~ a monitor protein in a cell,  
for example, a regulatory sequence, such as a promoter and enhancer; a selective  
gene, such as a drug resistance marker for detecting introduction of the nucleic acid  
10 into cells; etc.

This nucleic acid can be used for directly detecting a phosphorylation reaction  
in a cell by introducing the nucleic acid into the cell and directly expressing the above  
monitor protein as well as for producing the monitor protein. The monitor protein can  
be transported into a cell more effectively by introducing the nucleic acid encoding  
15 the monitor protein into the cell, because, in general, nucleic acids can be introduced  
into a cell more effectively than proteins. In addition, as described later, the above  
nucleic acid can be easily transported into a cell and prepared by harboring the nucleic  
acid in a vector.

20 4. A vector carrying a nucleic acid encoding a monitor protein

Any vector can be used. as long as ~~being~~ it is capable of self-replication and  
~~containing~~ contains a transcription initiation sequence. Any known plasmids which  
can be expressed in, for example, *E. coli*, yeast, plant cells, insect cells, mammalian  
cells, and such can be used. ~~Therefore, a~~ A plasmid can be ~~appropriately~~ selected

from these known plasmids, ~~corresponding to, for example, a cell to be examined as~~  
well as a corresponding cell.

5. A monitor protein for screening a kinase

5 A monitor protein can be used ~~for searching to search and screening to screen~~  
for an enzyme which phosphorylates a phosphorylation region in the monitor protein.  
An enzyme which phosphorylates a phosphorylation region can be screened by ~~acting~~  
reacting a monitor protein comprising the desirable phosphorylation region ~~on and~~ a  
test protein *in vivo* or *in vitro* and detecting phosphorylation of the monitor protein. In  
10 the case of screening *in vivo*, a vector carrying a nucleic acid encoding a monitor  
protein can be used. Any test proteins can be used. Natural protein libraries, artificial  
protein libraries, cDNA libraries thereof, and such can be used. Alternatively, for  
example, ribozyme libraries can be used.

15 6. A monitor protein for screening a compound which stimulates or inhibits  
phosphorylation

A monitor protein can be used for screening an accelerator ~~and or~~ inhibitor of  
phosphorylation or dephosphorylation enzyme activity ~~which phosphorylates or~~  
~~dephosphorylates a phosphorylation region in the monitor protein.~~ For example, by  
20 ~~contacting~~ a desired kinase is contacted with a monitor protein comprising the  
phosphorylation region to be phosphorylated ~~by the enzyme~~ in the presence of a test  
sample. A property change of the monitor protein is detected. Any test ~~samples~~  
sample can be used. Examples of ~~the a~~ test sample include cellular extracts,  
expression products of gene libraries, synthetic low molecular compounds, synthetic  
25 peptides, modified peptides, natural compounds. A compound which stimulates or

inhibits ~~the~~ a property change is screened ~~in comparison~~ by comparing with ~~the~~ a property change in the absence of the test sample. A compound which accelerates a property change of the monitor protein is ~~judged~~ identified as a compound which stimulates kinase activity, and a compound which inhibits a property change of the monitor protein is ~~judged~~ identified as compound which inhibits kinase activity. This enables isolation of a compound which stimulates or inhibits phosphorylation activity of the kinase. Combinations of a kinase to be used for screening and a phosphorylation region are not particularly limited.

Examples of kinases used for screening in the present invention (and phosphorylation sequences thereof) include, but are not limited to, A kinase (CREB phosphorylation sequence of SEQ ID NO: 1), G kinase (RKRS\*RAE; histone), AMP activation protein kinase (HMRSAMS\*GLHLVKRR; acetyl CoA carboxylase), calmodulin-dependent protein kinase II (PLRRTLS\*VAA; glycogen synthase) smooth muscle myosin light chain kinase (KKRAARATS\*NVFA; myosin light.chain), phosphorylase kinase (KRKQIS\*VRGSL; phosphorylase), C kinase (VRKRT\*LRRL; EGF receptor), v-Abl (RRLIEDAEY\*AARG; RR60<sup>SRC</sup>), and EGF receptor protein kinase (RREELQDDY\*EDD; erythrocyte band III), ~~but are not limited thereto (sequences and proteins in parentheses are substrate amino acid sequences and substrate proteins to be phosphorylated)~~ (R. B. Rearson et al., "Specific studies on protein kinase and phosphatase using synthetic peptides"; D. G. Hardie Eds., supervised by Hidaka, H., MEDSi Biological Experiment Series, Protein Kinase and Phosphatase, pp225-228, 1995, Medical Science International). Sequences and proteins in parentheses are substrate amino acid sequence and substrate proteins to be phosphorylated. These kinases are known to phosphorylate proteins comprising an

amino acid sequence other than the sequences identified above, ~~therefore~~ Therefore,  
an amino acid sequence at these substrate sites can be appropriately selected and used.

In the case of screening *in vivo*, a vector carrying a nucleic acid which encodes  
a monitor protein can be used. In this system, for example, compounds which  
5 stimulate or inhibit signal transduction leading to expression and activation of the  
kinase, and compounds which stimulate or inhibit dephosphorylation of a monitor  
protein as well as compounds which directly stimulate or inhibit the activity of the  
target kinase (an agonist and antagonist of the kinase) can be screened. Whether a  
compound directly acts on a kinase or not can be confirmed by an *in vitro* system  
10 using a purified kinase. In an *in vivo* system, for example, in the case of using GFP as  
a variable property region as described in Examples, property change of a monitor  
protein can be detected without crushing cells. Moreover, a property change of the  
monitor protein can be detected in real time in living cells. Effects of a test  
compound on phosphorylation can be examined in ~~details~~ detail in this manner.

15 ~~By these~~ These screenings, for instance, can conveniently screen for an  
inhibitor specific to a particular kinase ~~can be conveniently screened~~. Kinases have  
important functions in signal transduction in, for example, cellular proliferation,  
differentiation, and immunological responses. ~~For example~~ Thus, an isolated  
inhibitor ~~to be isolated~~ is expected to be able to be applied as a drug for preventing or  
20 treating various diseases involved in signal transduction.

#### Brief Description of the Drawings

Figure 1 shows the construction of pETIC vector.

Figure 2 shows. the purified recombinant protein separated by SDS-PAGE and  
25 stained with CBB.

Figure 3 shows the results of phosphorylation assay by A kinase.

Figure 4 shows the effect of the addition of A kinase on absorption wavelength for "A-Kinase Responsive Tracer (ART)" (derived from pETIC-ART).

Figure 5 shows the effect of addition of A kinase on absorption wavelength for  
5 "Kempart" (derived from pETIC-Kempart).

Figure 6 shows the effect of addition of A kinase on absorption wavelength for a negative control protein (derived from pETIC-1).

Figure 7 shows the construction of pCEP4.

Figure 8 shows the figure of ART in COS-7 living cells. (A) shows a BSGFP  
10 fluorescence image of ART expressed in a whole cells. (B) shows pseudo color images of BSGFP/RSGFP fluorescence intensity ratio in the cells in the same region as (A). The cells were treated with 5  $\mu$ M dibutyl-cAMP (db-cAMP) at hour 0. The bar indicates 30  $\mu$ m.

Figure 9 shows the fluorescence intensity ratios of ART in COS-7 cells  
15 (mean $\pm$ SEM, n=5) after the treatment with db-cAMP in the presence of H-89, which is a PKA inhibitor (the white bars), or the absence thereof (the black bars). Dibutyl-cAMP (db-cAMP), which is a cAMP analogue, was added at hour 0. R indicates the fluorescence intensity ratio of cellular images detected in the region of about 10  $\mu$ m X 10  $\mu$ m square at the various times. Rmin indicates the minimum fluorescence  
20 intensity ratio of the same cells.

#### Best Mode for Carrying Out the Invention

The present invention is illustrated in detail below with reference to the Examples, but is not construed as being limited thereto.

In these Examples, phosphorylation was detected by constructing a monitor protein, A-kinase Responsive Tracer ("ART")~~), by using a~~ The ART contained the phosphorylation sequence of CREB transcription factor (referred to as CREB phosphorylation sequence hereafter) as a phosphorylation region and RSGFP and  
5 BSGFP of *Aequorea victoria* as a measurement protein pair. SEQ ID NO: 1 shows the CREB phosphorylation sequence.

A monitor protein "Kempart" was ~~also~~ constructed in the same manner using the phosphorylation sequence (SEQ ID NO: 2) of kemptide instead of the CREB phosphorylation sequence, ~~and compared to the~~ The kempart monitor protein was  
10 compared to the monitor protein containing the above CREB phosphorylation sequence.

#### Example 1: Synthesis of a DNA fragment encoding a phosphorylation sequence

First, a DNA fragment encoding a phosphorylation sequence was synthesized  
15 to construct the above monitor protein. A DNA fragment encoding the CREB phosphorylation sequence (refer to as the CREB-DNA fragment, hereafter) was amplified by PCR using ~~the~~ oligonucleotide LCR-1B (SEQ ID NO: 3) as a template, and PCR-1K (SEQ ID NO: 5) and PCR-1A (SEQ ID NO: 6) as primers ~~for synthesis~~.

A DNA fragment encoding the phosphorylation sequence of kemptide  
20 (referred to as kemptide-DNA fragment hereinafter) was used as a control and was similarly amplified by PCR using ~~the~~ oligonucleotide Lke-1B (SEQ ID NO: 4) as a template, and ~~a primer set of~~ PKe-1K (SEQ ID NO: 7) and PKe-1A (SEQ ID NO: 8) as primers ~~for synthesis~~. Detailed manipulation ~~of~~ by PCR is as follows.

The PCR reaction mixture was prepared in a microtube (for 0.2 ml). The  
25 composition of the PCR reaction mixture was sterilized water (18.3  $\mu$ l), 10 X EXTaq



buffer (2.5  $\mu$ l), dNTP mixture (2.0  $\mu$ l), EXTaq polymerase (0.2  $\mu$ l) (Takara), template oligonucleotides (2.0 nmol/ml) (1.0  $\mu$ l), and primers (about 50 nmol/ml) (0.5  $\mu$ l each).

A microtube containing the above reaction mixture was set on a DNA  
5 amplifier (GeneAmp PCR System 2400, Perkin Elmer Japan) and amplified by PCR  
~~was carried out~~. PCR was performed under the following conditions: 1 cycle of a  
denaturation process (94°C for 30 sec); 40 cycles of a series of a denaturation process  
(94°C for 50 sec), an annealing process (57°C for 1 min), and an extension process  
(72°C for 1 min); a final extension process (72°C for 7 min); and cooling process at  
10 4°C.

After PCR, amplified fragments were confirmed by electrophoresis. A portion  
of the reaction mixture (10  $\mu$ l) was collected for electrophoresis and mixed with a  
~~buffer for an~~ electrophoresis buffer. This mixture was separated on a 10% agarose gel  
and stained with ethidium bromide to confirm the presence of target fragments.

15 The target fragments were excised with a razor and such, ~~and each of them~~  
~~was~~ transferred to a microtube (for 1.5 ml), and recovered using GeneClean III kit  
(BIO 1). Each collected DNA target fragment was dissolved into Tris EDTA (TE)  
buffer (100  $\mu$ l) and then purified. Purification was ~~conducted~~ performed by the  
following procedure. First, a mixture of phenol, chloroform, and isoamyl alcohol (25:  
20 24: 1) was added to the DNA solution and stirred. After ~~standing it~~ the solution was  
left to stand, the supernatant was removed, and ethanol precipitation was conducted.  
~~Supernatant~~ The supernatant was removed, and precipitated pellet was dissolved in  
sterilized water.

25 Example 2: Construction of a plasmid carrying a gene of a monitor protein

Each of the above DNA fragments was inserted into the pETIC vector (J. Biol. Chem., 272: 13270-13274, 1997) shown in Fig. 1. ~~This~~ The pETIC vector ~~carries~~ contains the RSGFP gene and BSGFP gene as a measurement protein pair, ~~and thus~~ Thus, a monitor protein gene ~~in which~~ contains a "RSGFP gene - a phosphorylation sequence - BSGFP gene" was ~~aligned~~ is constructed by inserting the above DNA fragments between the RSGFP ~~gene~~ and BSGFP gene.

First, the pETIC vector and the above two DNA fragments were digested with restriction enzymes KpnI and AgeI according to the standard method. After digestion, each ~~fragment~~ of the fragments and the linearized-vector were separated on a 10% agarose gel by electrophoresis. After separation, each target fragment was collected with GeneClean III kit in the same manner as ~~the~~ described above and purified. Each of these fragments was dissolved in TE buffer (10  $\mu$ l).

Next, CREB-DNA fragment or kemptide-DNA fragment was ligated to pETIC vector using the above DNA solution. Ligation was conducted with a DNA Ligation Kit Ver. 2 (Takara).

The CREB fragment solution (5  $\mu$ l) was mixed with the linearized pETIC vector solution (3  $\mu$ l), ~~and solution~~ Solution I (8  $\mu$ l) of the above kit was added thereto ~~to perform~~ and the reaction was performed at 16°C for 30 min. The kemptide fragment solution (5  $\mu$ l) was mixed with the pETIC vector solution (3  $\mu$ l) and solution I (8  $\mu$ l) of the above kit to perform ligation reaction in the same manner. The ligation ~~sample only with~~ was also prepared using TE buffer instead of the insert ~~was also prepared~~.

To collect a target plasmid from the above ligation reaction solution, the above ligation reaction solution (8  $\mu$ l) was mixed with JM109 competent cells (100  $\mu$ l), ~~and~~ transformation Transformation was conducted according to the standard method.

After transformation, the JM109 cells were spread on an agar medium containing kanamycin and cultured at 37°C overnight. After incubation, kanamycin-resistant colonies ~~grown that grew~~ were taken, and the plasmids in the cells were analyzed. ~~By this analysis, the cells~~ Cells containing plasmids in which the target fragments were inserted into pETIC vector were selected, and ~~the~~ plasmids were prepared from these cells. Among the plasmids prepared in this manner, the plasmid into which the CREB fragment was inserted and the plasmid into which the kemptide fragment was inserted were designated "pETIC-ART" and "pETIC-Kempart, respectively.

### 10 Example 3: Production of a monitor protein

Monitor proteins comprising the phosphorylation sequence derived from either CREB or kemptide were produced using the above "pETIC-ART" or "pETIC-Kempart," respectively. The pETIC vector, ~~a~~ the vector backbone for these plasmids, was prepared based on pET30a vector (Novagen), and has the functions of pET30a. Specifically, ~~in the pETIC vector;~~ contains a T7 promoter ~~is provided~~ upstream of the insert region, ~~and~~ Thus, expression of a target protein can be induced with IPTG in *E. coli* containing a T7 RNA polymerase gene downstream of a lacUV5 promoter, such as BL21 (DE3). The recombinant proteins ~~thus~~ expressed can ~~be~~ easily be collected with Ni agarose because a histidine tag (His-tag) is added to the proteins. The monitor proteins were produced using "pETIC-ART" and "pETIC-Kempart" by the following procedure based on the above principle.

BL21(DE3) competent cells were transformed with the above "pETIC-ART" or "pETIC-Kempart" plasmid using the same method as mentioned above. After transformation, colonies formed on the kanamycin-containing agar medium were taken, inoculated into 3 ml of LB medium (kanamycin 50 µg/ml) , and incubated at

37°C overnight to prepare the preculture medium. This preculture medium (2 ml) was transferred into a 2-L flask containing 500 ml of the same LB medium, and cultured at 37°C until absorbance at 600 nm reached 0.6. IPTG was added thereto (concentration: 1 mM) when absorbance at 600 nm was 0.6, and culturing was further  
5 performed at 23°C overnight.

The cultured medium was centrifuged (5,000 rpm, 10 min, 4°C), and cells were harvested. The collected cells were suspended in 10 ml of a buffer (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 2 mM benzamidine, 1 mM PMSF). The cells in the resulting suspension were disrupted by sonication on ice ~~for five times under the~~  
10 ~~condition~~ by delivery of a 30-second pulse. The extract was mixed with NP-40 and imidazole ~~with the~~ at a final concentration of 0.1% and 20 mM, respectively, stirred with a stirrer at 4°C, and centrifuged (10,000 rpm, 10 min) at 4°C to collect supernatant.

The collected supernatant was mixed with 500 µl of Ni-NTA-agarose (Qiagen)  
15 and stirred with a stirrer at 4°C for 60 min. After ~~stirred~~ stirring, the ~~above~~ Ni-NTA-agarose was washed with 2 ml of a binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM benzamidine, 1 mM PMSF, 0.1% NP-40, 20 mM imidazole) four times.

After ~~washed~~ washing, the agarose was mixed with 1 ml of an elution buffer  
20 (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 2 mM benzamidine, 1 mM -PMSF, 0.1% NP-40, 200 mM imidazole), stirred at 4°C with a stirrer for 30 min, and centrifuged to collect supernatant. This series of elution procedures was repeated twice. The collected eluate was mixed with glycerol at the final concentration of 20%, and stored at -80°C.

Ten microliters of the eluate collected in the above manner was subjected to ~~the~~ 10% acrylamide-gel electrophoresis to confirm the presence of a monitor protein. After electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB). The results were shown in Fig. 2. In Fig. 2, pETIC-1 (Romeser, V. A. et al, J. Biol. Chem., 272: 13270-13274) ~~was~~ is the pETIC vector into which a sequence encoding the  $\text{Ca}^{2+}$  calmodulin binding site was inserted, ~~and~~ It was used as a positive control in the preparation of the recombinant protein (RSGFP - the calmodulin binding site - BSGFP). "IPTG-" and "IPTG+" indicate the absence and the presence, respectively, of induction with IPTG during the incubation.

As shown in Fig. 2, ~~in the case of when~~ expression ~~induction~~ is induced with IPTG, ~~the protein shown as~~ a single band ~~was~~ is detected ~~in~~ with each of "pETIC-ART" and "pETIC-Kempart." The sizes of these proteins were similar to those of the proteins expressed by pETIC-1, which is a positive control. Therefore, the proteins expressed here were expected to be the target monitor proteins, namely RSGFP - CREB phosphorylation sequence - BSGFP 5 and RSGFP - kemptide phosphorylation sequence - BSGFP.

#### Example 4: Measurement of phosphorylation using $\gamma\text{-}^{32}\text{P}$

It was examined whether the phosphorylation sequences in the above two monitor proteins ~~would be~~ were phosphorylated ~~or not~~ in the an *in vitro* system. This examination was conducted using A kinase as ~~a~~ the kinase and [ $\gamma\text{-}^{32}\text{P}$ ]ATP as ~~a~~ the substrate according to the following procedure.

To prepare the reaction solution, 10 X kinase buffer (250 mM Hepes, pH 7.5, 100 mM  $\text{MgCl}_2$ , 10 mM DTT) (5  $\mu\text{l}$ ), A kinase (1  $\mu\text{l}$ ), [ $\gamma\text{-}^{32}\text{P}$ ]ATP (0.5  $\mu\text{l}$ ), and sterilized water (43  $\mu\text{l}$ ) were mixed. To this mixture, each eluate (0.5  $\mu\text{l}$ ) containing

each monitor protein was added, ~~and~~ The mixture containing the eluate was  
incubated at 30°C for 90 min to carry out phosphorylation reaction.

After the reaction, ~~whether each monitor protein was phosphorylated or not~~  
~~was examined by electrophoresis~~ was conducted to determine whether each monitor  
5 protein was phosphorylated. As a pre-treatment for electrophoresis, the reaction  
solution after the reaction (50 µl) was mixed with 2 X sample buffer (50 µl) and  
boiled for 3 min. After boiling, the solution (20 µl) was subjected to electrophoresis  
~~with~~ on the 10% acrylamide gel. After electrophoresis, the gel was exposed to films  
to detect the signal ~~signals~~ of  $\gamma$ -<sup>32</sup>P (Fig. 3).

10 As shown in Fig. 3, signals were detected both for the monitor protein  
derived from "pETIC-ART" and for the monitor protein derived from  
"pETIC-Kempart." Specificity of these signals ~~were~~ was obvious by the fact that  
signals were not detected for pETIC-1-derived protein which did not contain any  
phosphorylation sequence. The respective positions of the signals detected for  
15 "pETIC-ART" and "pETIC-Kempart" corresponded to the size of the monitor proteins  
expressed by the plasmids shown in Fig. 2. These results confirmed that the two  
monitor proteins produced in Example 3 maintained the phosphorylation sequences  
and that the sequences were phosphorylated.

### Example 5: Measurement of phosphorylation by fluorescence change

One end of the phosphorylation sequence in the above two monitor proteins was ligated to RSGFP and the other to BSGFP. Fluorescence change due to interference of RSGFP and BSGFP at the each side by phosphorylation of the phosphorylation sequence in these two monitor proteins was examined.

As a reaction solution, 10 X kinase buffer (20  $\mu$ l), ATP (100 mM) (2  $\mu$ l), and sterilized water (168  $\mu$ l) were mixed and the above monitor proteins (5  $\mu$ g/ $\mu$ l) (10  $\mu$ l) were added to this mixture. Moreover, to this reaction solution, 0, 1, 2, or 4  $\mu$ l of A kinase were added.

The prepared reaction solution was incubated at 30°C for 30 min. Changes in the range of ~~wavelength~~ wavelengths absorbed at ~~the a~~ range of ~~wavelength~~ wavelengths between 430 nm and 520 nm were measured in the incubated reaction solution with a fluorescence spectrophotometer (Nippon Bunnko). Figures 4, 5, and 6 show the results for fluorescence changes of "ART" (derived from pETIC-ART) , "Kempart- (derived from pETIC-Kempart), and pETIC-1 (negative control) derived recombinant protein proteins, ~~which is a negative control~~, respectively. In each of the above figures, ~~the difference in the~~ different amounts of A kinase added ~~was expressed with the~~ to each reaction is shown as a different lines line.

As shown in Fig. 4, in "ART," a difference in the absorbance patterns was observed ~~between the case that~~ for reactions in which A kinase was not added and ~~the case that~~ reactions in which A kinase was added. When A kinase was not added, the stronger absorbance was observed at 500 nm. As the amount of added A kinase increased, the absorbance at 450 nm was enhanced. This indicates that ~~conformation a~~ conformational change was generated by phosphorylation of the CREB

phosphorylation sequence, ~~and thereby allowing~~ RSGFP and BSGFP at the either end ~~could~~ of the CREB sequence to interfere with each other, emitting fluorescence.

In contrast ~~in "Kempart"~~ (Fig. 5), although phosphorylation of "Kempart" was confirmed ~~in the phosphorylation measurement~~ using [ $\gamma$ - $^{32}$ P]ATP in Example 3  
5 above, no fluorescence change was observed in the above-described wavelength range (Fig. 5) as ~~in~~ seen with the negative control (Fig. 6). From these results, it was expected that the phosphorylation sequence of kemptide was phosphorylated, but the phosphorylation was not accompanied ~~with~~ by a conformational change.

Thus, it has been revealed that the above "ART" can be used for, for instance,  
10 detecting a phosphorylation reaction using the above-described fluorescence change as an indicator. ~~In~~ An *in vivo* system can be constructed by introducing pETIC-ART, which expresses this "ART", into cells. Moreover, the above "ART" can be used not only for detecting a phosphorylation reaction, but also for screening kinases and screening accelerators and inhibitors of phosphorylation activity.

15 On the other hand, it was shown that there was a difference between the phosphorylation sequence of CREB ~~phosphorylation sequence~~ and kemptide ~~phosphorylation sequence~~ in terms of the presence and absence of conformational change at the time of phosphorylation. This finding indicates that RSGFP and BSGFP can be used for analyzing whether a conformational change occurs in phosphorylation  
20 sequences upon phosphorylation. Specifically, it was shown that a conformational change at the time of phosphorylation can be observed by inserting any phosphorylation sequence between RSGFP and BSGFP and by performing both phosphorylation measurements using [ $\gamma$ - $^{32}$ P]ATP and ~~those based on~~ fluorescence change.



#### Example 6: *In vivo* measurement of Phosphorylation

##### 1) Structure and construction of pCEP4-ART

The pETIC-ART plasmid and the mammalian expression vector pCEP4 (Invitrogen) plasmid vector (Fig. 7) were digested with the restriction enzymes HindIII and XhoI and were electrophoresed on a 10% agarose gel. ~~to collect the~~ The target bands ~~with~~ were collected using a GeneClean III kit. The collected DNA was dissolved in 10 µl of TE<sub>7.2</sub> ~~and 8 µl~~ Eight microliters of the DNA solution was mixed with 8 µl of solution I of DNA Ligation Kit Ver. 2 (Takara) to perform the ligation reaction at 16°C for 30 min. Eight microliters of the solution were mixed with 100 µl of JM109 *E. coli* competent cells, and the cells were transformed. The cells were spread on an ampicillin plate and cultured at 37°C overnight. Colonies were then taken and cultured in 3 ml of LB medium (ampicillin 50 µg/ml) at 37°C overnight. Two milliliters of the overnight cultured solution were added into 500 ml of LB medium (ampicillin 50 µg/ml) in a 2-liter flask, and cultured at 37°C overnight. The cultured *E. coli* was collected and the plasmid DNA (pCEP4-ART) was collected with Qiagen Tip (Qiagen).

##### 2) Introduction of plasmid DNA into COS-7 cells

One microgram of pCEP4-ART plasmid DNA was added to 100 µl of serum-free DMEM medium, ~~and 4 µl~~ Four microliters of lipofectAMINE ~~reagent~~ reagent (GIBCO BRL) were added to another 100 µl of serum-free DMEM medium. These two DMEM media were mixed ~~with each other~~ together and ~~stood~~ allowed to stand for 30 to 40 min at room temperature. Then, ~~the~~ medium in a glass-bottomed culture dish (MatTek) ~~in which~~ containing COS-7 cells, which had been passage-cultured ~~from~~ the day prior to DNA introduction, was replaced with 800 µl of

serum-free DMEM medium<sub>2</sub> ~~and the~~ The mixture of plasmid DNA and lipofectAMINE reagent was added thereto ~~to culture~~ and the cells were cultured for 3 hours. The medium was then replaced with 1 ml of DMEM medium containing 10% bovine serum, and culturing was performed for 2 days.

5

### 3) Imaging of phosphorylation

COS-7 cells transfected with pCEP-4 ART were spread on a cover slip (a diameter of 14 mm) of a glass-bottomed culture dish (MatTek). The cells were incubated at 30°C for 48 hours and rinsed twice with Ringer's buffer (150 mM NaCl, 10 4 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM Hepes, Ph 7.4, and 5.6 mM glucose)<sub>2</sub>. The cells were then ~~and~~ kept in the buffer in the dark at room temperature. Fluorescence intensity was measured with excitation light at 380 nm by detecting with 440 ± 20-nm and 520 ± 20-nm band pass filters using a 100 X oil immersion objective lens (OLYMPUS UV-Fluor), a Quantix CCD camera (Roper Scientific), and an 15 IPLab Spectrum image processor (Scanalytics). ~~Exposure duration for Cells were~~ exposed to the excitation light at 380 nm ~~was~~ for a duration of 0.2 sec, and at exposure ~~interval was~~ intervals of 20 sec. An image passed through the 440 ± 20 nm filter was detected, and after 5 seconds, an image passed through 520 ± 20 nm filter was detected. Cells were induced with dibutyryl-cAMP (db-cAMP), a cAMP 20 analogue, by adding 500 µl of Ringer's buffer containing 10µM db-cAMP to ~~a~~ the culture dish containing 500-µl of Ringer's buffer.

### 4) Results

~~Observation for~~ The intracellular distribution of "ART" ~~revealed that "ART"~~ 25 in COS-7 cells was dispersed ~~in the COS-7 cells~~ (Fig. 8A). The concentration of

intracellular cyclic-adenosine monophosphate (cAMP) ~~was~~ increased ~~with~~ in the cells  
in the presence of db-cAMP, an analogue of cAMP, to activate A kinase, ~~and~~ A  
fluorescence change in the cells was also measured in real time with a cooled CCD  
camera (Fig. 8B). db-cAMP was added at ~~the time of~~ 0 sec. Fluorescence intensity  
5 ratios in the cellular image indicate that values of fold induction (fluorescence  
intensity ratio) increased depending on the time after the addition of db-cAMP. The  
increase in fold induction was transmitted from the side of the cell membrane to the  
nucleus, coinciding with the phenomenon that an active form of A kinase moves from  
the cytoplasm to the nucleus after the increase of cytoplasmic cAMP. The average  
10 fold induction increased with time after db-cAMP stimulation. This indicates that  
"ART" can be used as phosphorylation monitor system in a living cell.

To examine whether "ART" is ~~actually~~ phosphorylated in COS-7 cells  
depending on A kinase, intracellular A kinase was inhibited with H-89, ~~which is a~~  
selective inhibitor of A kinase, ~~to perform the~~ The experiment for db-cAMP  
15 stimulation was performed in the same manner as Fig. 8. The same experiment as  
described above was conducted except that the cells were pre-treated about for 2  
hours with DMEM containing 10  $\mu$ M H-89 and that, ~~then,~~ the medium was then  
replaced with Ringer's buffer containing 10  $\mu$ M H-89. Fluorescence change of "ART"  
in the cells was measured with a cooled CCD camera in real time (Fig. 9).  
20 Comparison of fluorescence intensity ratios in the region of a 10  $\mu$ m X 10  $\mu$ m range  
indicates that the fluorescence intensity ratio was low in the presence of H-89,  
showing that inhibition of A kinase activity can be observed in a living cell in real  
time.

As shown above, using the monitor proteins of the present invention, the  
25 system for monitoring phosphorylation *in vivo* was constructed. Using this system,

phosphorylation can ~~be~~ easily be monitored ~~under the~~ in an environment more similar to physiological conditions. Moreover, it was shown that the effects of inhibitors on kinase can ~~be~~ easily be monitored using this system. Therefore, this system is useful for screening a compound which stimulates or inhibits phosphorylation.

5

#### Industrial Applicability

In the present invention, an analysis system ~~in~~ was developed which uses no radioactive isotopes ~~are used~~ and ~~that~~ is applicable to *in vivo* measurement ~~has been developed for~~ of protein phosphorylation. The analysis system of the present  
10 invention can be used not only ~~for the detection of~~ to detect a phosphorylation reaction, but also for screening a kinase and for screening a compound which stimulates or inhibits phosphorylation.